

Laboratory Procedure Manual

Analyte: **Hepatitis D**

Matrix: **Serum**

Method: **International Immunodiagnostics**

Method No.:

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as performed by: *Hepatitis Branch
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National Center for Infectious Diseases*

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Important Information for Users

The National Center for Infectious Diseases periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the item listed in the following table:

File Name	Variable Name	SAS Label
HEPHD_d	LBXHD	Hepatitis D

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The International Immunodiagnostics HDV Ab assay is a competitive enzyme immunoassay (ELISA) for the determination of antibodies to Hepatitis D Virus in plasma and serum with a "one-step" methodology. The Hepatitis Delta Virus or HDV is a RNA defective virus and infection with HDV only occurs in the presence of acute or chronic HBV infection. The detection of HDV total antibodies allows the classification of the illness and the monitoring of the serconversion event.

Anti-HDV antibodies, if present in the sample, compete with a virus-specific polyclonal IgG, labeled with peroxidase (HRP), for a fixed amount of rec-HDV coated on the Microplate, in a "one step" incubation. The concentration of the bound enzyme on the solid phase is inversely proportional to the amount of anti-HDV antibodies in the sample and its activity is detected by adding the chromagen/substrate in the second incubation. The concentration of HDV-specific antibodies in the sample is determined by means of a cut-off value that allows for the semi-quantitative detection of anti-HDV antibodies.

2. SPECIAL SAFETY PRECAUTIONS

Test kits for anti-HDV contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance that these reagents are entirely noninfectious. Therefore, they should be treated as though capable of transmitting disease. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and HBV. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method because of infectious contamination hazards. Place all plastic and glassware contaminated with serum in a plastic autoclave bag for disposal. We recommend Biosafety Level 2 containment and practice as described in CDC/NIH publication #88-8395 for handling test specimens and kit reagents (1).

Material Safety Data Sheets (MSDSs) are available through the National Center for Infectious Diseases (NCID) Local Area Network (LAN). Risk is minimal because of the small quantity of chemicals, the packaging of the chemicals, and limited handling of chemicals by the operators using the test kits.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

- a. Raw data are transcribed manually from an instrument readout sheet into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results, and functions within SQL Server software (Microsoft, Redmond, WA) with a Visual Basic (Microsoft, Redmond, WA) user interface. Test values are compared with a cutoff value calculated from the controls. Results are expressed as "positive" or "negative" Other information in the database may typically include the HRL identification number, the specimen number, the date collected, the date tested and results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer. Electronically stored data are backed up routinely.
- b. Finished data are reviewed by the lab supervisor. After each NHANES container is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the results to the SQL Server along with other NHANES IV data.
- c. Files stored on the CDC Local Area Network (LAN) are automatically backed up nightly to tape by CDC Data Center staff.
- d. Documentation for data system maintenance is maintained with printed copies of data records for 2 years.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES;
CRITERIA FOR SPECIMEN REJECTION**

- a. Specimens submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (W. Kuhnert, 10/02).
- b. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.
- c. Specimens may be serum, recalcified plasma, or plasma. Serum specimens may be collected using regular red-top or serum-separator Vacutainers.
- d. Required sample volume is 10 μ L for the assay; 1.0 mL will permit repeat analyses as well as other testing.
- e. Specimens should be stored in plastic vials and sealed tightly to prevent desiccation of the sample.
- f. Serum or plasma samples are collected aseptically to minimize hemolysis and bacterial contamination.
- g. Samples are stored in labeled 2 mL Nalgene cryovials or equivalent.
- h. Serum is best stored frozen, and freeze/thaw cycles should be kept to a minimum. Store samples at 4-8°C for no more than 5 days.
- i. For storage >5 days, samples are held at -20°C. Samples held in long-term storage at -20°C are indexed in the database for easy retrieval.
- j. Specimens are rejected if contaminated, hemolyzed, or stored improperly. However, rejection is done only after consultation with NCHS.
- k. Avoid multiple freeze/thaw cycles.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF
INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION,
CALIBRATORS (STANDARDS), AND CONTROLS**

a. Instrumentation

- (1) Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (70% ethanol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample or the components of the kit. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/- 2%.
- (2) The ELISA incubator has to be set at +37°C (tolerance of +/-0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.

- (3) The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit controls/calibrator and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 300 µl/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested when the efficiency of the washer or the washing step needs to be improved. In order to set correctly the right number of the washing steps, it is recommended to run an assay with the kit controls/calibrator and well characterized negative and positive reference samples, and check to match the values reported below in the sections "validation of test" and "assay performances". Regular calibration of the volumes delivered and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instruction of the manufacturer.
- (4) The ELISA Microplate reader has to be equipped with a reading filter of 450 nm and ideally with a second filter (620 – 630 nm) for blanking purposes. Its standard performances should be (a) bandwidth ≤ 10 nm; (b) absorbance range from 0 - ≥ 2.0 ; (c) linearity to ≥ 2.0 ; repeatability $\geq 1\%$. Blanking is carried out on the well identified in the section "assay procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer's instructions.

b. Other Materials

- (1) International Immunodiagnostics HDV Ab (total) kit, cat no. 288. Each kit includes the following:
 - a. Microplate – 8x12 microwell strips coated with purified recombinant HDV and sealed into a bag with desiccant.
 - b. Negative Control – 1x1.0ml /vial. Contains human serum proteins and 0.3 mg/ml gentamicine sulphate as preservative.
 - c. Positive Control – 1x1.0ml/vial. Contains human serum proteins and anti-HDV antibodies and 0.3 mg/ml gentamicine sulphate as preservative.
 - d. Wash buffer concentrate – 1x60ml/bottle. 20x concentrated solution to be diluted up to 1200ml distilled water before use. Contains phosphate buffer, Tween 20 and Kathon GC as preservative.
 - e. Enzyme conjugate – 1x0.5ml/vial. 20X concentrated solution. Contains Horseradish peroxidase conjugated antibody specific to HDV in presence of 0.3 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservative.
 - f. Enzyme conjugate diluent – 2x10ml/vial. The diluent contains a proteic buffered solution for the dilution of the concentrated conjugate in presence of 0.3 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservative.
 - g. Chromogen – 1x8ml/vial. Contains a buffered solution of stabilized hydrogen peroxide or H₂O₂.
- (2) Calibrated micropipettes in the range of 10 – 100 µl. a disposable plastic tips.

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- (3) EIA grade water (double distilled or deionized charcoal treated to remove oxidizing chemicals used as disinfectants.
- (4) Timer with 60 minute range or higher
- (5) Absorbent paper tissue.
- (6) Calibrated ELISA Microplate thermostatic incubator (dry or wet) set at 37°C
- (7) Calibrated ELISA Microplate washer.
- (8) Vortex or similar mixing tools.

c. Reagent Preparation

Washing solution – The concentrated solution has to be diluted 20x in ELISA grade water before use.

Conjugate – Dilute the concentrated conjugate 1:20 with the Conjugate Diluent. Mix gently or vortex before use. Prepare only the volume necessary for the test; the diluted solution is not stable.

Chromogen/Substrate – About 5 minutes before use, prepare this reagent in a disposable plastic tube, according to needs, by missing 1 volume of Chromogen with 1 volume of Substrate. Prepare only the volume necessary for the test; the mixture is stable at room temperature for 4 hr if protected from light.

Example of preparation:

Component	24 Tests	48 Tests
Conjugate	70 ml 20x Enzyme Conjugate 1.4 ml conjugate Diluent	140 ml 20x Enzyme Conjugate 2.8 ml Conjugate diluent
TBM/H ₂ O ₂	2.0 ml Chromogen 2.0 ml Substrate	4.0 ml Chromogen 4.0 ml Substrate

d. Standards Preparation

This method does not employ conventional calibrators or standards. Calibration is based on the results of defined positive and negative controls. Positive and negative control reagents are supplied with each test kit. The assay cutoff value is calculated from values obtained from these controls.

e. Preparation of Quality Control Materials

Kit positive and negative controls are prepared and quality monitored by the manufacturer.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Calibration Curve

No calibration curve is generated by the user as part of this method. Calibration of instruments is either automatic or is carried out periodically by contracted service personnel.

b. Verification

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A check is performed on the controls/calibrator any time the kit is used in order to verify whether the expected OD450nm or S/co values have been matched in the analysis.

Ensure that the following parameters are met:

Check	OD450nm
Blank Well	< 0.100 OD450nm
Negative Control (NC)	> 1.000 OD450nm after blanking
Positive Control (PC)	OD450nm < NC/10

If the parameters are not met, do not proceed any further and perform the following checks:

Problem	Check
Blank Well > 0.100 450nm	That the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control (NC) < 1.000 450nm after blanking Coefficient of variation > 30%	<ol style="list-style-type: none"> 1. that the washing procedure and the washer settings are as validated in the pre-qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of negative control); 4. that no contamination of the negative control or of the wells where the control was dispensed has occurred due to positive samples, to spills or to the enzyme conjugate; 5. that micropipettes have not become contaminated with positive samples or with the enzyme conjugate; 6. that the washer needles are not blocked or partially obstructed.
Positive Control OD450nm > NC/10	<ol style="list-style-type: none"> 1. that the procedure has been correctly performed; 2. that no mistake has occurred during the distribution of the control (dispensation of negative control instead of positive control); 3. that the washing procedure and the washer settings are as validated in the pre-qualification study; 4. that no external contamination of the positive control has occurred.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preliminaries

- (1) Check the expiration date of the kit printed on the external label. Do not use if expired.
- (2) Check that the liquid components are not contaminated by visible particles or aggregates. Check that the Chromogen is colorless or pale blue by aspirating a small volume of it with a sterile plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the Microplate, is not punctured or damaged.
- (3) Dilute all of the content of the 20x concentrated Wash Solution as described.
- (4) Dilute the 20x concentrated Enzyme Conjugate, as reported before.

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- (5) Allow all other kit components to reach room temperature (about 1 hr) and then mix gently on vortex all liquid reagents.
- (6) Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
- (7) Check that the ELISA reader is turned on or ensure it will be turned on as least 20 min before reading.
- (8) Check that the micropipettes are set to the required volume.
- (9) Check that all other equipment is available and ready to use.

b. Sample Preparation

- (1) Bring serum specimens to 20-25 °C.
- (2) Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix the samples gently before testing.

c. Assay Procedure

- (1) Place the required number of strips in the microplate holder. Leave the 1st well empty for the operation of blanking. Store the other strips in the bag in the presence of the desiccant at 2-8°C, sealed.
- (2) Pipette 50 µl of negative control in duplicate, 50 µl positive control in singlicate and then 50 µl of each of the samples. Add 50 µl diluted enzyme conjugate in all wells except for the blanking A1 well. Then incubate the plate at 37°C for 90 minutes. *Important note: be careful not to touch the inner surface of the well with the pipette tip when dispensing the Enzyme Conjugate. Contamination may occur.*
- (3) Wash the microplate with an automatic washer by delivering and aspirating 300 µl/well of diluted washing solution for 5 times with soaking. *Important note: the washing procedure is essential for the assay to provide reliable and precise results.*
- (4) Pipette 100 µl Chromogen/Substrate mixture in each well, the blank well included. Then incubate the microplate at room temperature for 20 minutes. *Important note: do not expose to strong light as a high background might be generated.*
- (5) Pipette 100 µl of Sulphuric Acid into each well to stop the enzymatic reaction, using the same pipette sequence as in step 4. Then measure the color intensity of the solution in each well using a micro well reader at 450nm filter (reading) and possibly at 620-630nm (blanking), blanking the instrument on the 1st well.

Important notes:

- *If the second filter is not available, ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Fingerprints could generate false positive result on reading.*
- *Reading should ideally be performed immediately after the addition of the Stop Solution, but definitely no longer than 20 minutes afterwards. Some self oxidation of the chromogen can occur leading to a higher background.*

e. **Recording of Data**

(1) Quality control data

Raw data are transcribed manually from the instrument readout sheets into a computerized database.

(2) Analytical results

For EIA, raw data are expressed as absorbance values. Raw data are transcribed manually from the instrument readout sheet into a computerized database.

Results are interpreted according to the following table:

S/Co	Interpretation
> 1.2	Negative
0.8 – 1.2	Equivocal
< 0.8	Positive

- A negative result indicates that the patient has not been infected by HDV
- Any patient showing an equivocal result should be re-tested on a second sample taken 1-2 weeks after the initial sampling.
- A positive result is indicative of HDV infection and therefore the patient should be treated accordingly.

f. **Replacement and Periodic Maintenance of Key Components**

- (1) Incubator temperature, quality of water used, refrigerator temperature, freezer temperature, and room temperature are monitored and documented on a weekly basis.
- (2) All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

- (1) Results are calculated by means of a cut-off value determined with the following formula:

$$\text{Cutoff} = (\text{NC/PC})/5$$

9. REPORTABLE RANGE OF RESULTS

Final results are expressed qualitatively as positive or negative for the presence of anti-HDV antibody in the sample. No quantitative results are determined.

10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been validated for use in the Hepatitis Reference Laboratory for epidemiologic studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-art.

The presence or absence of anti-HDV is determined by comparing the absorbance value of the sample to the cutoff value. This cutoff value is calculated from the negative and positive control absorbance values as explained in the calculations section. Specimens having absorbance values lower than or equal to the cutoff value are considered reactive by the criteria of the test kit.

An external positive anti-HDV control serum is purchased from Blackhawk Biosystems, Inc. and used in each testing run. When tested by using the International Immuno-diagnostics assay, the final anti-HDV in-house control (IHC) reagent must generate a signal-to-cutoff ratio that falls within a specified range. This is developed by calculating a range of ± 3 standard deviations from the mean after performing multiple runs on separate days. This range is re-calculated for each lot purchased and is specified within the DMS.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

- a. By definition, if controls do not conform to specifications, reject the test results and retest all samples. Do not use data from nonqualifying test runs.
- b. If the substrate blank falls outside the expected range, repeat the run.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

No interfering substances have been identified. The International Immuno-diagnostics anti-delta EIA is limited to the detection of anti-delta in human serum or plasma.

Specimens containing sodium azide may give false-positive reactions and should not be tested.

13. REFERENCE RANGES (NORMAL VALUES)

Normal human serum should be negative for hepatitis anti-delta antibodies.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may remain at 20-25 °C for 4 hours during preparation and testing.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

In case of system failure, specimens may be refrigerated at 4-8 °C for 5 days. For longer periods, specimens should be stored at -20°C until the system is functioning.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

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Test results are documented through the lab management database (Section 3). Generally, studies that are conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be electronic or in printed form. All electronically held data are backed up routinely.

In long-term storage, specimens are arranged by study group. The storage location of each sample is listed with the test data. Standard record-keeping means (e.g., electronic, mainframe data files, laboratory notebook, and floppy disks) should be used to track specimens. We recommend that records, including QA/QC data and duplicate records, be maintained for 10 years.

Only numerical identifiers (e.g., case ID numbers) should be used.

19 SUMMARY STATISTICS AND GRAPHS

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

REFERENCES

1. U.S. Department of Health and Human Services. Biosafety in microbiological and biomedical laboratories. HHS publication (NIH) 88-8395. Washington: U.S. Government Printing Office, 1988.